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Rapid visual detection of *Treponema pallidum* using the RPA-CRISPR/Cas12a system

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Syphilis, caused by *Treponema pallidum*, is a sexually transmitted infection that has re-emerged globally over the past decade, posing significant public health challenges. Conventional diagnostic methods are limited by lengthy processing times, operational complexity, and moderate sensitivity, highlighting the urgent need for rapid, sensitive, and user-friendly detection strategies. In this study, we developed a visual detection platform for *T. pallidum* DNA by integrating recombinase polymerase amplification (RPA) with CRISPR/Cas12a technology. The assay can be completed within one hour, with results directly interpreted via fluorescence readout. It demonstrated a detection limit as low as 11.34 copies/μL and high specificity, accurately distinguishing *T. pallidum* without cross-reactivity with common blood-borne pathogens, including HIV, HBV, HCV, and DENV. The clinical sample verification showed a consistency rate of 96.6% with the actual diagnosis. To enhance suitability for point-of-care applications, the RPA-CRISPR/Cas12a system was further adapted to a lateral flow assay (LFA) format, achieving a detection sensitivity of 5.56×10^2 copies/μL while minimizing reliance on specialized instrumentation. Overall, this platform provides a rapid, sensitive, and robust approach for point-of-care syphilis diagnosis and offers a reference framework for detecting other pathogenic organisms.

Keywords Treponema pallidum, Recombinase polymerase amplification, CRISPR/Cas12a, Visualization detection

Syphilis, a sexually transmitted infection (STI) caused by the spirochete *Treponema pallidum subsp. pallidum* has undergone a significant global resurgence in recent decades[1,2]. Since the early 2000s, syphilis incidence has risen markedly worldwide, exacerbating the overall burden of STIs and posing substantial challenges to public health infrastructure and socioeconomic stability[3]. Epidemiological data indicate that approximately 8 million new syphilis cases were reported globally in 2022, with congenital syphilis cases alone reaching an alarming 1.5 million in 2023. These figures underscore persistent gaps in transmission prevention, maternal screening, and neonatal care[4]. Early detection and timely treatment are essential for mitigating mortality, averting severe sequelae, and enhancing long-term patient outcomes and quality of life[5].

Current syphilis diagnosis predominantly relies on serological antibody assays, categorized into non-treponemal tests (NTTs) and treponemal tests (TTs)[6]. NTTs detect immunoglobulin M and G antibodies against lipids released from damaged host cells or cardiolipin components of *T. pallidum*[7]. Their sensitivity varies by disease stage, ranging from 62% to 78% in primary and late syphilis to 97%-100% in secondary syphilis[8,9]. Despite their simplicity and cost-effectiveness, which facilitate widespread use in screening programs, NTTs are susceptible to false positives in conditions such as pregnancy, autoimmune disorders, and concurrent infections. In contrast, TTs-including the *Treponema pallidum* particle agglutination assay (TPPA), *Treponema pallidum* hemagglutination assay (TPHA), enzyme-linked immunosorbent assay (ELISA), and chemiluminescence immunoassay (CLIA)-target antibodies against *T. pallidum*-specific antigens, providing superior sensitivity and specificity[10,11]. These assays are particularly effective for confirmatory diagnosis in early syphilis, addressing NTT limitations during this phase[12]. However, a key drawback of TTs is their inability to distinguish between

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active and resolved infections, as anti-treponemal antibodies often persist lifelong, rendering them unsuitable for monitoring treatment efficacy or disease activity.

To enhance diagnostic precision, molecular approaches such as *Treponema pallidum* polymerase chain reaction (TP-PCR) have been developed, targeting genomic loci including polA[13], tp47[14], and 23 S rRNA[15]. Among these, polA and tp47 are the most commonly validated and implemented targets in clinical laboratories worldwide. Although TP-PCR offers high accuracy, its adoption is constrained by requirements for specialized equipment, technical expertise, and elevated costs[16]. Consequently, there remains a critical need for rapid, sensitive, specific, and accessible diagnostic tools to bolster syphilis control and curb the epidemic.

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) system, initially acclaimed for genome editing, has been adapted for molecular diagnostics[17]. Systems such as CRISPR/Cas12a[18], CRISPR/Cas12b[19], and CRISPR/Cas13a[20] leverage programmable nucleic acid recognition for highly specific target detection, establishing CRISPR-Cas as a versatile platform for nucleic acid-based diagnostics.

In recent years, isothermal nucleic acid amplification technology has been widely used in the detection of bacteria and viruses, including loop-mediated isothermal amplification (LAMP)[21], recombinase polymerase isothermal amplification(RPA)[22], helicase-dependent isothermal amplification (HDA)[23], etc. The reaction temperature of LAMP was 60–65 °C, and the amplification time required was 1 h, which took a long time. At present, studies have reported that LAMP technology has been successfully applied to the detection of *Treponema pallidum* and *Haemophilus ducreyi*[24,25]. Compared with LAMP technology, RPA technology can achieve rapid amplification of double-stranded DNA at 37 °C without the need for thermal cycling equipment[26].

Integrating RPA with CRISPR-Cas enhances sensitivity and specificity, facilitating detection of low-abundance targets in resource-limited settings[27]. Relative to traditional PCR methods, RPA-CRISPR/Cas12 platforms offer improved performance while obviating the need for costly instrumentation and advanced facilities. Their adaptability supports diverse readout formats, including fluorescence, lateral flow strips, and naked-eye visualization, making them ideal for point-of-care and field applications[28–30].

In this study, we describe the development, optimization, and validation of a rapid, highly sensitive, and specific syphilis detection assay based on an RPA-CRISPR/Cas12a platform. Results are interpretable via three modalities: naked-eye observation (NEO), lateral flow assay (LFA), and fluorescence-based detection (FBDA). This approach enables efficient identification of *T. pallidum* and serves as a foundational framework for advancing point-of-care diagnostics for syphilis and other infectious pathogens.

Materials and methods

Materials and reagents

All nucleic acid sequences used in this study, including the recombinant tp47 plasmid, RPA primers, nucleic acid templates from Human Immunodeficiency Virus (HIV, GenBank: D86068.1), Hepatitis B virus(HBV, GenBank: MT426913.1), Hepatitis C virus (HCV, GenBank: KJ439771.1), and Dengue virus (DENV, GenBank: M14931.2). CRISPR RNAs (crRNAs), the fluorescent ssDNA reporter, and the lateral flow strip reporter were synthesized by Aiji Biotechnology Co., Ltd. (Guangzhou, China). EnGen Lba Cas12a (Cpf1) was obtained from New England Biolabs (Ipswich, MA, USA), and TwistAmp Basic DNA Amplification Kits were purchased from TwistDx Ltd. (Cambridge, UK). Tiosbio Cas12/13-specific nucleic acid test strips were acquired from Beijing Baoying Tonghui Biotechnology Co., Ltd. (Beijing, China). Human serum samples for clinical validation were provided by our institutional laboratory. Informed consent was obtained from all subjects and/or their legal guardian(s). All procedures involving human specimens were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Review Committee of the Ninth People's Hospital of Dongguan (Approval No. 3, 2022).

Plasmid construction and copy number calculation

The tp47 gene (GenBank: M88769.1) was selected as the target for syphilis detection and cloned into the pBluescript II SK(-) vector using standard recombinant DNA techniques. Plasmid DNA copy number was determined using the formula: DNA copy number per microliter = [(6.02 × 10^23) x (plasmid concentration, in nanograms per microliter) x 10^{-9}]/[(fragment length, in nucleotides) x660], where 6.02×10^{23} represents Avogadro's number, and 660 is the average molecular weight of a base pair in Daltons.

CrRNA and RPA primer design

CrRNA sequences of 41 nucleotides were designed using CRISPR RGEN Tools (http://www.rgenome.net/cas-designer/). The specificity of each crRNA targeting tp47 was validated via NCBI BLAST. RPA primers were subsequently designed based on the tp47 sequence encompassing the crRNA binding region, following the guidelines in the TwistAmp Assay Design Manual. Three candidate primer pairs were evaluated for specificity using NCBI BLAST, and the optimal primer pair was selected based on amplification efficiency and product size confirmed by agarose gel electrophoresis (Table 1).

RPA amplification

RPA was performed using the TwistAmp Basic Kit following the manufacturer's instructions. The reaction mixture (total volume 47.5 µL) comprised 29.5 µL resuspension buffer, 2.4 µL each of forward and reverse primers (10 µM), 1 µL target nucleic acid template, and one enzyme pellet, with nuclease-free water added to reach the final volume. After thorough mixing, 2.5 µL of magnesium acetate (280 mM) was added to initiate the reaction. The mixture was briefly centrifuged and incubated at 39 °C for 30 min.

Name	Oligonucleotide sequence (5'-3')
tp47F1	GTTCCTCATGAATTAAAAGGGATTGCAAAG
tp47R1	AAAAACTATCCTCAGTGAGCATTGTCTTAAGG
tp47F2	CGAGGAATACAAGATTACGAACGTAAAGGT
tp47R2	CAGAAAAACTATCCTCAGTGAGCATTGTCT
tp47F3	TAAGACAATGCTCACTGAGGATAGTTTTTC
tp47R3	ACATAGTCGATGAACTCACGGTGCGACAGC
crRNA	UAAUUUCUACUAAGUGUAGAUUGCACGUAAGGUAAGCAGCA
ssDNA-FQ	6-FAM- TTATT-BHQ1
ssDNA-FB	6-FAM- TTATT-Biotin

Table 1. The oligonucleotide sequences for the primer, crRNA, and SsDNA reporter.

Fluorescence detection assay based on RPA-CRISPR/Cas12a

For fluorescence-based detection, 10 µL of the RPA-amplified product was combined with 250 nM crRNA, 100 nM EnGen Lba Cas12a (New England Biolabs, USA), 200 nM fluorescent reporter probe and 1× NEBuffer 2.1 in a total volume of 30 µL. The mixture was incubated at 37 °C for 30 min. Fluorescence was monitored in real time at 1 min intervals using a real-time PCR system (Applied Biosystems). Fluorescence was also visualized under blue light (470 nm) using a Tanon MINI Space 1000 Gel Imaging System after reaction completion.

Lateral flow assay based on RPA-CRISPR/Cas12a

For lateral flow detection, the RPA amplification and CRISPR/Cas12a reaction were performed as described above, except that the reporter probe was labeled with FAM and biotin. Following the reaction, the mixture was diluted with 70 µL of ddH_2O, and a lateral flow strip was inserted into the tube. Results were interpreted visually within 10 min. After 10 min of incubation at room temperature, the signal values of T and C lines were recorded. ImageJ software was used to analyze and quantify the results. Apply the scanning function of a smartphone for image collection.

Evaluation of sensitivity and specificity

The sensitivity of the RPA-CRISPR/Cas12a detection system was assessed using serial dilutions of recombinant tp47 plasmid ranging from 1 ng/µL to 1 fg/µL, prepared with nuclease-free water. Nuclease-free water alone served as the no-template control (NTC). By performing linear regression analysis of the logarithm of fluorescence values and concentrations, the standard curve is established, and the regression equation is obtained. In this method, 3 times the mean fluorescence signal of the negative control sample was used as the detection threshold. Finally, the detection threshold is substituted into the above standard curve equation to calculate the corresponding target concentration, which is defined as the minimum detection limit of the method.

The specificity of the assay was evaluated by testing nucleic acids from Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Dengue Virus (DENV), which can present clinical symptoms similar to *Treponema pallidum* infection, following the detection procedures described above.

Clinical sample evaluation

In this study, 30 peripheral venous blood samples from individuals with suspected syphilis were collected. Genomic DNA was extracted from all samples using a fully Automated nucleic acid extraction system (GeneRotex96, Xi'an Tianlong Technology Co., Ltd.) following the operating manuals and syphilis detection was performed using the RPA-CRISPR/Cas12a assay. The diagnostic performance of this assay was subsequently compared with the laboratory reference standard.

The current laboratory reference standard for syphilis diagnosis primarily relies on serological testing, including *Treponema pallidum* specific antibody detection by chemiluminescent immunoassay (CLIA) in combination with non-*Treponema pallidum* testing using the Toluidine red unheated serum test (TRUST), to determine infection status. During RPA-CRISPR/Cas12a testing, the true clinical status of each specimen was blinded to the operator to minimize bias. A positive result was defined using the assay's established limit of detection as the cutoff threshold. Upon completion of testing, sample identities were unblinded, and the RPA-CRISPR/Cas12a results were systematically compared with the reference diagnostic outcomes to evaluate assay concordance and diagnostic accuracy.

Statistical analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Statistical comparisons were conducted using unpaired t-tests with GraphPad Prism 9. Differences were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Assay workflow for the tp47-targeted RPA-CRISPR/Cas12a-based rapid visual detection of *T. pallidum*

The working principle of the tp47-targeted RPA-CRISPR/Cas12a visual detection system is schematically illustrated in Fig. 1. Briefly, the target *T. pallidum* gene fragment is first amplified using RPA. At a constant

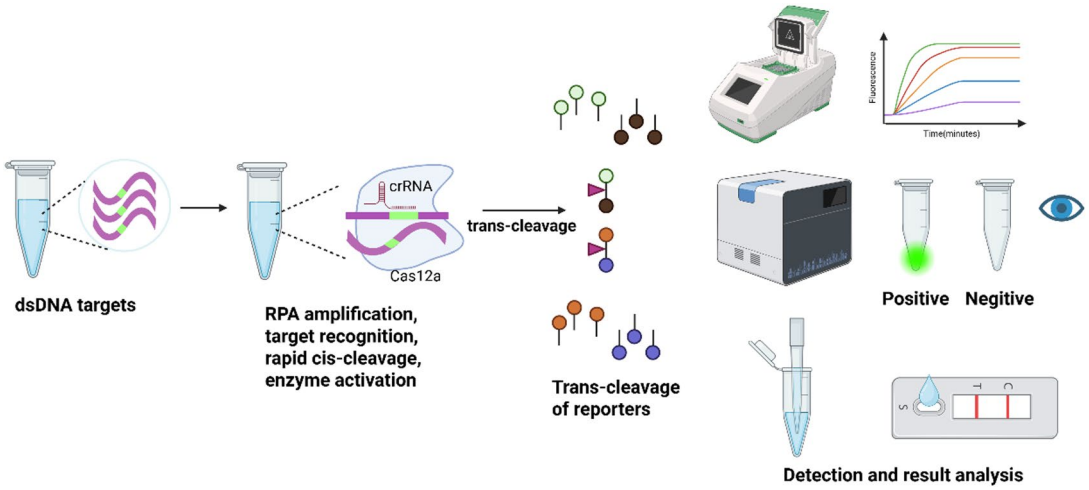

Fig. 1. RPA-CRISPR/Cas12a detection principle. The detection principle of the RPA-CRISPR/Cas12a system for the TP detection. "Created with BioRender.com".

temperature of 39 °C, recombinase proteins form nucleoprotein complexes with specific oligonucleotide primers, which subsequently scan double-stranded DNA for homologous sequences. Upon recognition of complementary target regions, strand invasion occurs, enabling primer extension by DNA polymerase and rapid exponential amplification of the target sequence within approximately 30 min.

Following RPA amplification, the amplified products are subjected to CRISPR/Cas12a detection. The Cas12a-crRNA complex recognizes the target DNA through sequence-specific base pairing adjacent to a protospacer adjacent motif (PAM). Upon target binding, Cas12a is activated and acquires robust trans-cleavage activity, resulting in the nonspecific cleavage of single-stranded DNA (ssDNA) reporter probes[31]. Fluorophore-quencher (FQ)-labeled ssDNA reporters generate a fluorescence signal upon cleavage, while biotin-labeled ssDNA reporters (FB) enable visual readout through lateral flow chromatography.

Collectively, this integrated RPA-CRISPR/Cas12a platform supports multiple detection modalities, including real-time fluorescence measurement, direct visual observation under blue light, and lateral flow assay-based interpretation. The assay provides a rapid, sensitive, and instrument-flexible strategy for the detection of *T. pallidum*, highlighting its potential utility in point-of-care and resource-limited diagnostic settings[32].

Feasibility validation and primer screening of the RPA-CRISPR/Cas12a-based rapid visual detection system

To experimentally validate the feasibility of the RPA–CRISPR/Cas12a detection system, five reaction conditions (R1-R5) were established, each lacking a critical component of the assay. R1 contained the complete reaction mixture, including target DNA, Bst DNA polymerase, Cas12a, and crRNA. In contrast, R2-R5 were formulated by individually omitting the target DNA, Bst DNA polymerase, Cas12a, or crRNA, respectively. Under UV illumination, only the complete reaction (R1) generated a robust fluorescence signal, whereas all incomplete reactions (R2-R5) exhibited no detectable or only negligible background fluorescence (Fig. 2A). These findings were further corroborated by real-time fluorescence monitoring, which revealed a marked signal increase exclusively in R1, while fluorescence levels in R2-R5 remained at baseline throughout the reaction (Fig. 2B). The absence of fluorescence in the incomplete reaction groups is consistent with the mechanistic requirement for Cas12a activation, which depends on both sequence-specific target recognition by the crRNA-Cas12a complex and the presence of amplified target DNA. Collectively, these results confirm the high specificity of the RPA-CRISPR/Cas12a system for Treponema pallidum detection and demonstrate a minimal risk of nonspecific activation or false-positive signals. The concordance between endpoint fluorescence visualization and real-time kinetic analysis highlights the robustness and reliability of this detection platform.

Based on prior evidence supporting its diagnostic relevance, the tp47 gene of T. pallidum was selected as the molecular target for nucleic acid detection. Accordingly, three pairs of RPA primers were designed and synthesized targeting conserved regions of the tp47 sequence. Comparative analysis of amplification efficiency demonstrated that the F3/R3 primer pair produced superior amplification yield and specificity relative to the other primer sets (Fig. 2C). Therefore, the F3/R3 primer pair was selected for all subsequent RPA amplification assays.

Optimization of reaction parameters for the RPA-CRISPR/Cas12a detection system

To achieve optimal analytical performance of the RPA-CRISPR/Cas12a assay, several key reaction parameters including RPA amplification time, Cas12a concentration, crRNA concentration, and ssDNA reporter concentration, were systematically optimized. RPA amplification time was evaluated first, as it directly determines the abundance of target DNA available for downstream Cas12a activation. Fluorescence intensity increased progressively during the initial amplification phase and reached a plateau at approximately 30 min,

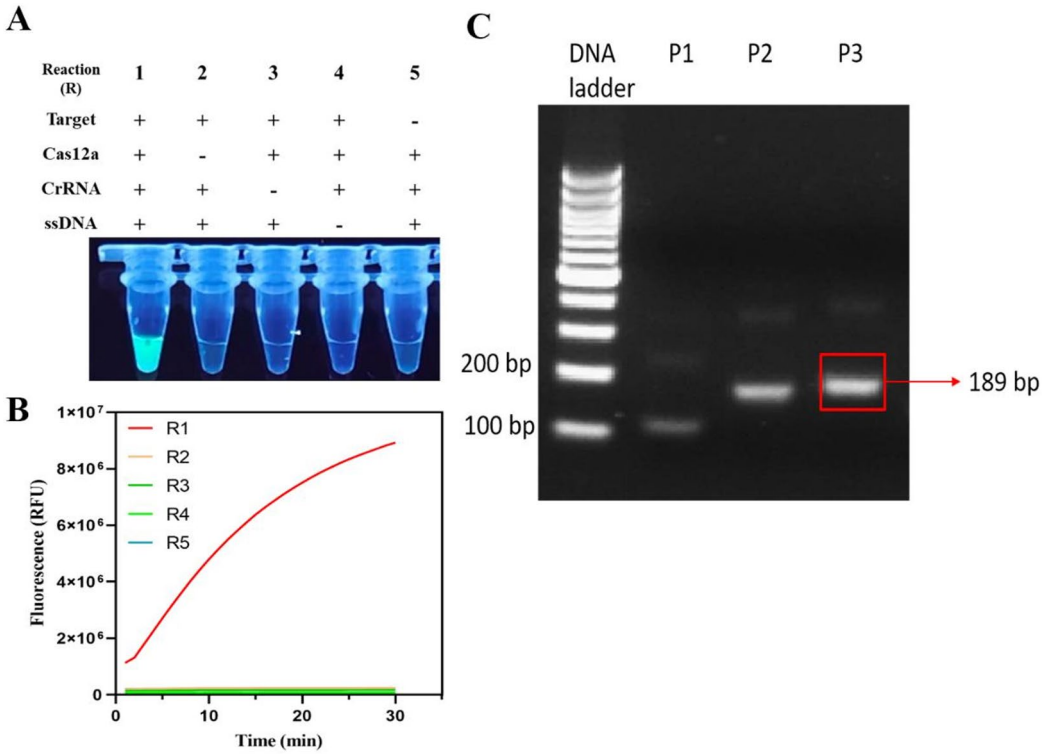

Fig. 2. Feasibility verification and primer screening based on RPA-CRISPR/Cas12a system. (**A**) Feasibility analysis of the RPA-CRISPR/Cas12a system for TP detection. "+" and "−" represent the "presence" and "absence" of the corresponding components in each reaction, respectively. Fluorescence images of five reactions (R1-R5) with various components were captured using a scanner. (**B**) Real-time fluorescence curves caused by isothermal amplification with five reactions (R1-R5) for the detection of the tp47 gene. (**C**) Specificity of RPA primers for the *tp*47 gene was visualized by 1% agarose gel electrophoresis (P1:F1R1, P2:F2R2, P3:F3R3).

indicating saturation of target amplification. Based on these kinetics, an RPA incubation time of 30 min was selected for all subsequent experiments (Fig. 3A-B).

Subsequently, the concentrations of Cas12a and crRNA were optimized to maximize trans-cleavage efficiency and signal output. Cas12a was tested at final concentrations of 25, 50, 100, 200, and 250 nM, while crRNA concentrations ranged from 50 to 500 nM. Among the tested conditions, the combination of 100 nM Cas12a and 250 nM crRNA produced the strongest and most stable fluorescence signal, and was therefore identified as the optimal configuration (Fig. 3C-F).

Collectively, these optimization steps established a robust and well-balanced set of reaction conditions that ensure efficient target amplification, maximal Cas12a activation, and reliable signal readout, thereby enhancing both the sensitivity and reproducibility of the RPA-CRISPR/Cas12a detection platform.

Sensitivity and specificity evaluation of the RPA-CRISPR/Cas12a fluorescence assay

Under optimized reaction conditions, the analytical sensitivity of the RPA-CRISPR/Cas12a fluorescence assay was evaluated using a series of tenfold dilutions of the tp47 plasmid, ranging from 1 fg/test to 1 ng/test. Fluorescence intensity increased in a concentration-dependent manner across the tested range. A strong linear relationship was observed between fluorescence signal intensity and the logarithmic concentration of target DNA from 10 fg/test to 100 pg/test, with a correlation coefficient (R^2) of 0.99, indicating excellent quantitative performance (Fig. 4A-C).

Based on three independent measurements of the negative control (NC), the limit of detection (LOD) was calculated using the mean signal plus three standard deviations, yielding an LOD of 2.04 fg/test, corresponding to approximately 11.34 copies/μL as determined by DNA copy number conversion. The analytical specificity of the assay was further assessed using the tp47 target in parallel with nucleic acids from clinically relevant pathogens associated with overlapping clinical manifestations, including HIV, HBV, HCV, and DENV. Robust fluorescence signals were detected exclusively in reactions containing the tp47 target, whereas all non-target viral samples and negative controls produced no detectable or only background-level signals in both real-time and endpoint fluorescence analyses (Fig. 4D-E). Notably, even at a high target input of 1 ng/test, the fluorescence intensity of tp47 remained markedly higher than that of all non-target controls, underscoring the high specificity of the assay and its strong discriminatory capability for *Treponema pallidum* detection.

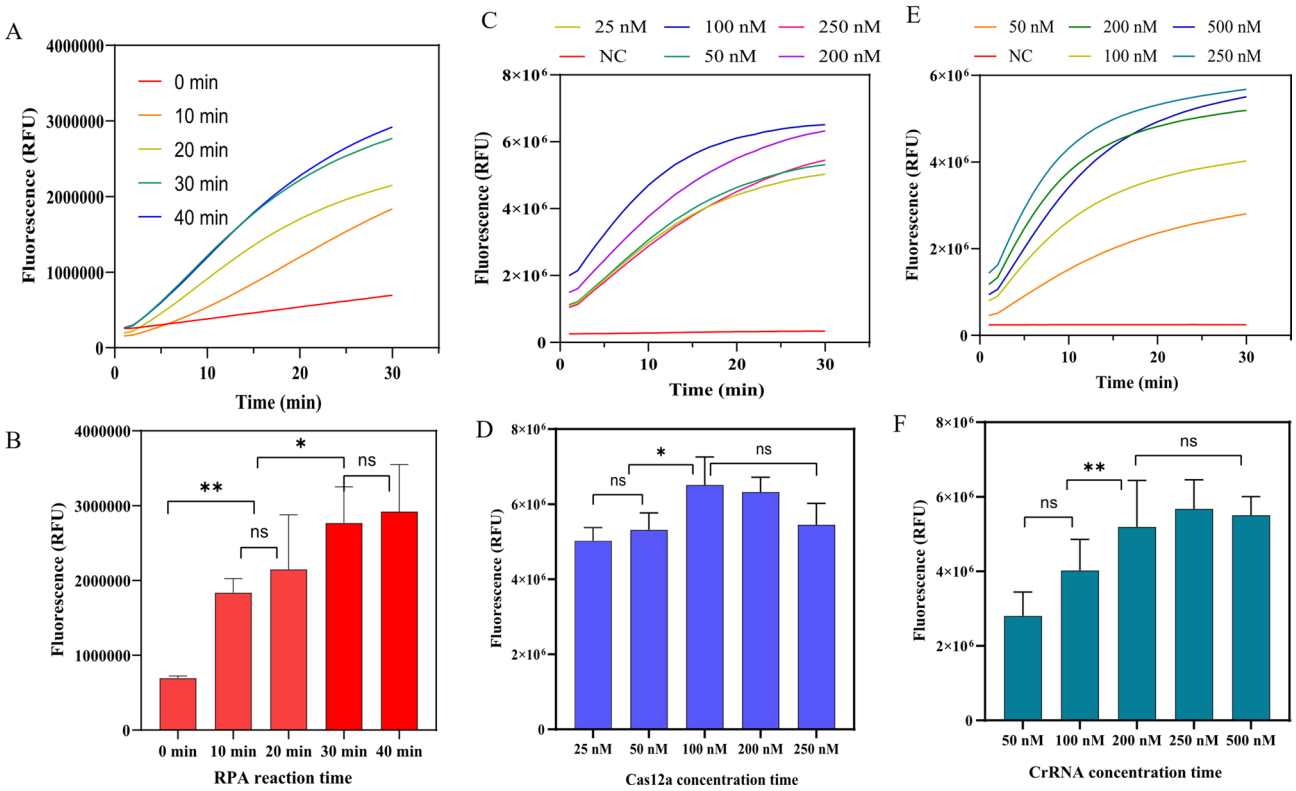

Fig. 3. Optimization of reaction parameters for the RPA-CRISPR/Cas12a system. (**A**) Real-time fluorescence curves of RPA reactions at different reaction times in the presence of target DNA (5.56×10^5 copies/μL recombinant plasmid tp47). (**B**) Fluorescence of different RPA reactions at times ranging from 0 min to 40 min. (**C**) Real-time fluorescence curves of Cas12a at different concentrations in the presence of target DNA (5.56×10^6 copies/μL recombinant plasmid tp47 as target DNA). (**D**) The fluorescence value of RPA-CRISPR/Cas12a with various concentrations of Cas12a (5.56×10^6 copies/μL recombinant plasmid tp47 as target DNA). (**E**) The fluorescence value of RPA-CRISPR/Cas12a with various concentrations of CrRNA (5.56×10^6 copies/μL recombinant plasmid tp47 as target DNA). (**F**) Real-time fluorescence curves of CrRNA at different concentrations in the presence of target DNA (5.56×10^6 copies/μL recombinant plasmid tp47 as target).

Clinical validation

To evaluate the diagnostic performance of the developed RPA-CRISPR/Cas12a fluorescence assay, a total of 30 clinically characterized serum samples were analyzed. A positive cut-off value was defined as three times the maximum fluorescence intensity observed in the negative control samples. Under this criterion, fluorescence signals were successfully detected across all tested samples. Representative results are shown in Fig. 5, in which samples ST1, ST2, ST3, ST4, ST7, ST9, ST10, ST11, and ST13 exceeded the predefined threshold and were classified as syphilis-positive by the assay.

Following completion of the fluorescence analysis, sample identities were unblinded and compared with the corresponding clinical diagnoses, which served as the reference standard. The RPA-CRISPR/Cas12a assay demonstrated a diagnostic accuracy of 96.6% across the tested cohort (Table 2), highlighting its high concordance with established clinical diagnostic methods and supporting its potential utility for rapid and reliable syphilis detection in clinical settings.

Optimization and evaluation of an RPA-CRISPR/Cas12a–based lateral flow assay for point-of-care syphilis detection

To adapt the RPA-CRISPR/Cas12a detection platform for POCT applications in syphilis diagnosis, recombinase polymerase amplification (RPA) was integrated with the CRISPR/Cas12a system and a LFA readout. The feasibility of the combined RPA-CRISPR/Cas12a-LFA system was first assessed using a component omission strategy[33]. A distinct test line was observed only when all essential reaction components were present, confirming that signal generation strictly depended on the integrity of the complete reaction system (Fig. 6A).

Subsequently, four critical reaction parameters: RPA amplification time, Cas12a protein concentration, crRNA concentration, and ssDNA reporter concentration were systematically optimized to maximize assay performance. The optimal conditions were determined to be a 20 min RPA amplification, 100 nM Cas12a, 50 nM crRNA, and 200 nM ssDNA reporter, which together produced the strongest and most consistent test line signals (Fig. 6B-E).

Under these optimized conditions, the analytical sensitivity of the RPA-CRISPR/Cas12a-LFA was evaluated using serial dilutions of the tp47 recombinant plasmid. As shown in Fig. 6F, the test-to-control (T/C) line

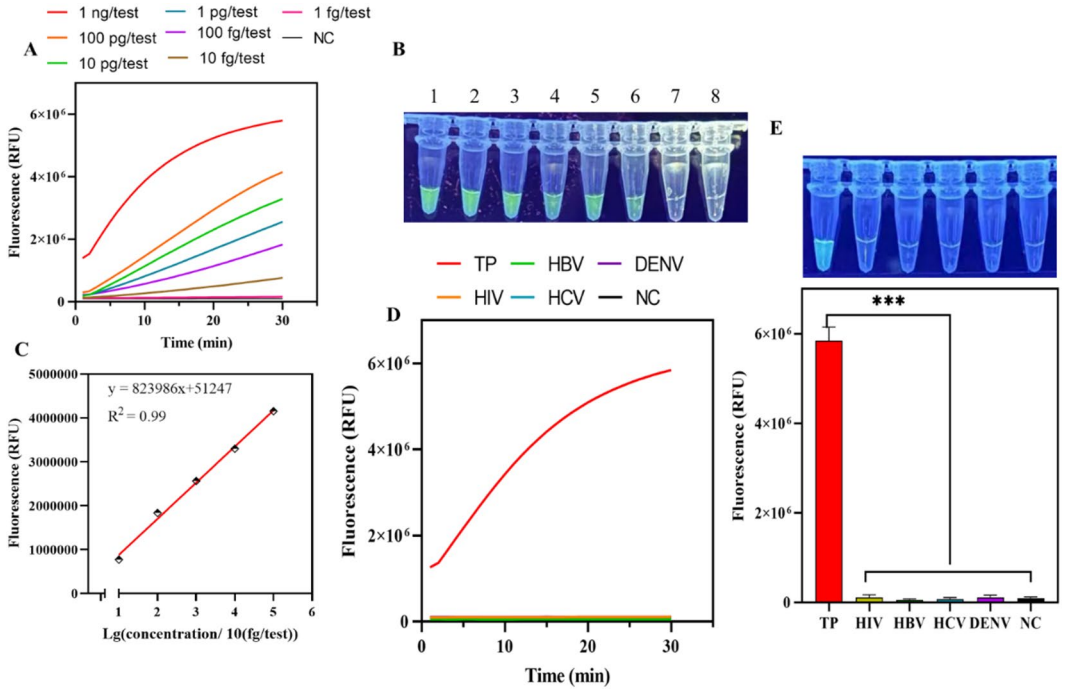

Fig. 4. Methodological evaluation of RPA-CRISPR/Cas12a based on fluorescence detection. (**A**) Real-time fluorescence curve detected from the RPA-CRISPR/Cas12a System for recombinant plasmid tp47 at different concentrations ranging from 1 fg/test to 1 ng/test (5.56 copies/ul-5.56×10^6 copies/ul). (**B**) Fluorescence images of eight reactions (R1-R8, R1: 1 ng/test, R2: 100 pg/test, R3: 10 pg/test, R4: 1 pg/test, R5: 100 fg/test, R6: 10 fg/test, R7: 1 fg/test, R8: NC.) were captured using a scanner. (**C**) Linear relationship between the fluorescence and the logarithm of target DNA concentrations. Error bars represent standard deviation, $n = 3$. (**D**) Real-time fluorescence curves detected from the RPA-CRISPR/Cas12a System for TP, HIV, HBV, HCV, and DENV. (**E**) Photograph (top) and bar graph depicting fluorescence intensity for TP, HIV, HBV, HCV, and DENV.

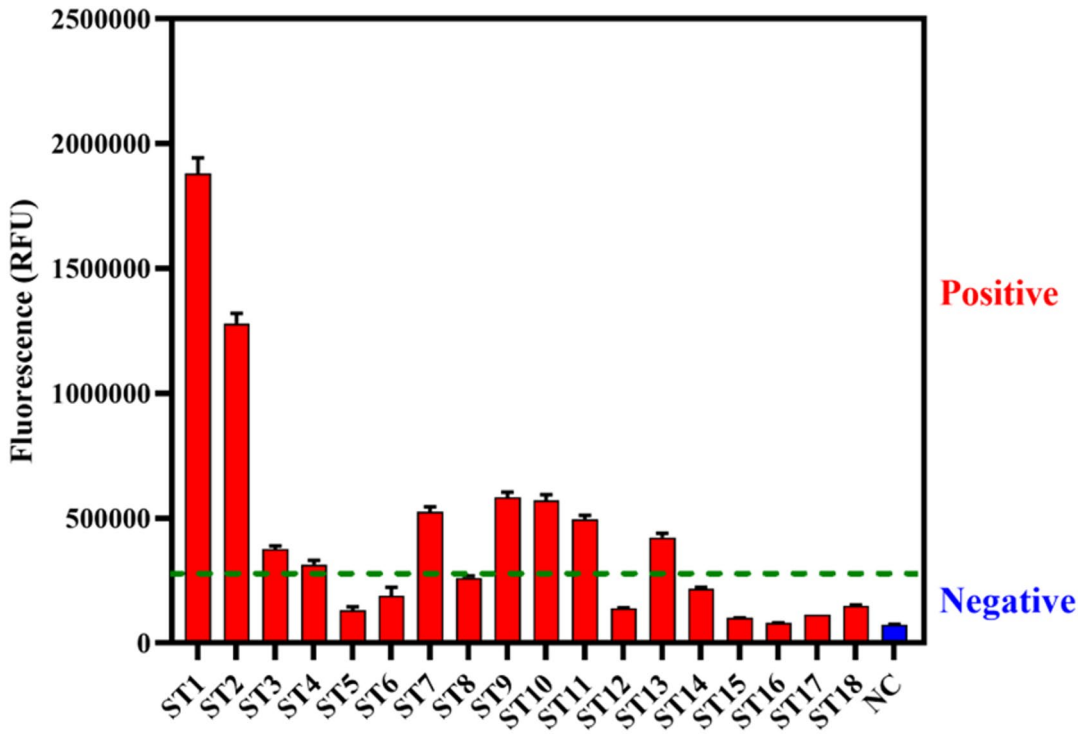

Fig. 5. Fluorescence detection results of some clinical samples. The fluorescence signal was detected by the CRISPR/Cas12a system for 30 min. NC: negative control.

		Clinically practical diagnosis			Diagnostic accuracy
		Disease	Healthy controls	Total	
RPA- CRISPR Cas12a (fluorescence)	Positive	9	0	9	96.6%
	Negative	1	20	21	
	Total	10	20	30	

Table 2. Diagnostic analysis of RPA-CRISPR Cas12a fluorescence test results.

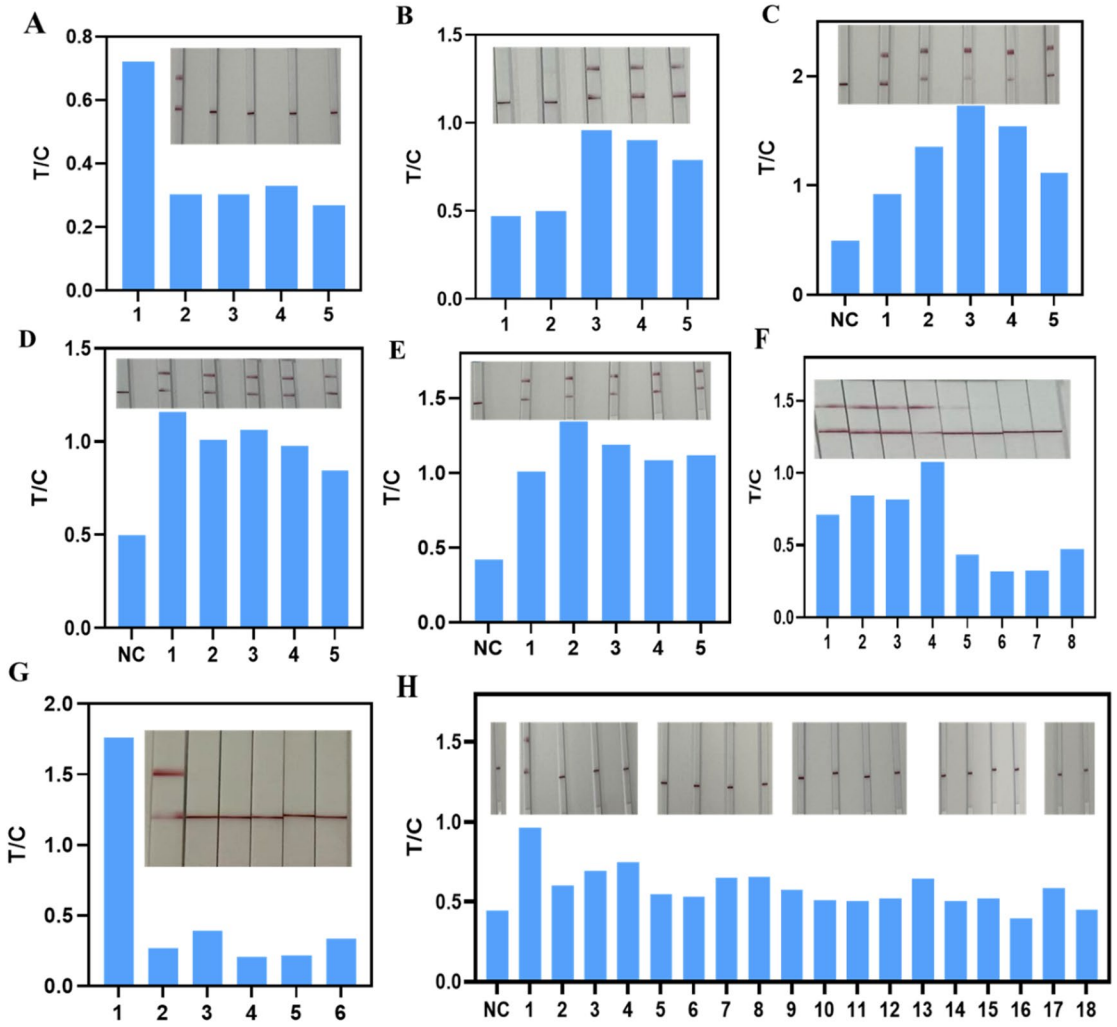

Fig. 6. Evaluation and application of an RPA-CRISPR/Cas12a-based lateral flow assay for point-of-care syphilis detection. **(A)** Feasibility verification of the RPA-CRISPR/Cas12a-LFA for *Treponema pallidum* detection (1: Complete reaction system; 2: Without Cas12a; 3: Without crRNA; 4: Without ssDNA reporter; 5: Without target). **(B)** Optimization of amplification time for RPA-CRISPR/Cas12a-LFA detection of *Treponema pallidum* (1: 0 min; 2: 10 min; 3: 20 min; 4: 30 min; 5: 40 min). **(C)** Optimization of Cas12a protein concentration for RPA-CRISPR/Cas12a-LFA detection of *Treponema pallidum* (1: 25 nM; 2: 50 nM; 3: 100 nM; 4: 200 nM; 5: 250 nM). **(D)** Optimization of crRNA concentration for RPA-CRISPR/Cas12a-LFA detection of *Treponema pallidum* (1: 50 nM; 2: 100 nM; 3: 200 nM; 4: 250 nM; 5: 500 nM). **(E)** Optimization of ssDNA reporter concentration for RPA-CRISPR/Cas12a-LFA detection of *Treponema pallidum* (1: 100 nM; 2: 200 nM; 3: 250 nM; 4: 400 nM; 5: 500 nM). **(F)** Sensitivity evaluation of the RPA-CRISPR/Cas12a-LFA for *Treponema pallidum* detection (1: 1 ng/test; 2: 100 pg/test; 3: 10 pg/test; 4: 1 pg/test; 5: 100 fg/test; 6: 10 fg/test; 7: 1 fg/test; 8: NC). **(G)** Specificity evaluation of the RPA-CRISPR/Cas12a-LFA for *Treponema pallidum* detection (1: TP, *Treponema pallidum*; 2: HIV; 3: HBV; 4: HCV; 5: DENV; 6: NC). **(H)** Clinical sample validation of the RPA-CRISPR/Cas12a-LFA for *Treponema pallidum* detection (representative results).

intensity ratio increased progressively with increasing target concentrations and was readily distinguishable from the negative control. The limit of detection (LOD) was defined as the lowest target concentration yielding a visible test line that could be reliably differentiated from the background signal. Using this criterion, the LOD for the tp47 plasmid was determined to be 100 fg/test, corresponding to approximately 5.56×10^2 copies/μL.

The analytical specificity of the assay was further assessed using nucleic acids from clinically relevant pathogens, including TP, HIV, HBV, HCV, and DENV. As illustrated in Fig. 6G, a distinct test line was observed exclusively in the TP-containing samples, while no detectable signal was generated by non-target pathogens, confirming the high specificity of the RPA-CRISPR/Cas12a-LFA.

Finally, the clinical applicability of the assay was preliminarily evaluated using 30 clinical serum samples. Representative LFA results are shown in Fig. 6H, in which one syphilis-positive specimen was correctly identified. Collectively, these results demonstrate the feasibility and translational potential of the RPA-CRISPR/Cas12a-LFA as a rapid, instrument-free POCT platform for syphilis detection. Although the diagnostic performance observed in this initial clinical evaluation was lower than that achieved with the fluorescence-based assay, further optimization of reaction conditions, strip design, and signal amplification strategies is expected to substantially improve assay sensitivity and accuracy.

Discussion

In this study, we developed an RPA-CRISPR/Cas12a detection system for the rapid diagnosis of syphilis. Over the past two decades, the incidence of syphilis has steadily increased, and its diverse clinical manifestations often overlapping with other infections, pose significant challenges to accurate diagnosis and timely treatment[34]. Currently, syphilis detection relies primarily on combined serological testing (*Treponema pallidum*-specific and non-*Treponema pallidum* assays), which serves as a standard approach for screening, diagnosis, and monitoring disease progression and treatment efficacy. Nucleic acid amplification assays, such as conventional PCR, provide direct detection of *T. pallidum* DNA[6]. For instance, Zhou et al. employed both conventional PCR and droplet digital PCR to detect spirochete DNA in plasma samples from patients at different stages of syphilis, demonstrating high detection efficiency[35]. However, PCR-based methods require expensive instrumentation and trained personnel, limiting their utility for large-scale screening, particularly in resource limited or grassroots settings.

Compared with traditional amplification methods, the RPA-CRISPR/Cas12a system offers a simpler workflow and requires minimal equipment, significantly reducing both detection cost and turnaround time. These advantages facilitate on-site pathogen detection. The tp47 gene has been widely selected as a target for *T. pallidum* detection in previous studies[36]. tp47 encodes a cytoplasmic membrane protein involved in cell wall synthesis, making it a stable and specific target for nucleic acid-based assays[37]. RPA is an isothermal amplification technique that has gained popularity due to its rapid amplification, high sensitivity, and operational simplicity. Nonetheless, RPA is prone to primer-dimer formation and nonspecific amplification[38]. CRISPR/Cas12a, as a molecular detection tool, overcomes some of these limitations through its trans-cleavage activity: upon recognition and binding of crRNA to a complementary double-stranded DNA target, Cas12a is activated and cleaves fluorescent reporter probes, releasing a detectable signal[39]. Several RPA-CRISPR/Cas12a-based viral detection assays have been reported. For example, Li et al. developed two RPA-based assays for monkeypox virus: a fluorescence-based RPA (F-RPA) with an LOD of 15.32 copies/μL, and a vertical flow bar RPA (VF-RPA) with an LOD of 8.53 copies/μL[40]. Ren et al. reported an RPA-CRISPR/Cas12a dengue virus assay with an LOD of 91.7 copies/test[41], while Lin et al. described a rapid detection method for Plasmodium parasites, achieving an LOD of 1 copy/μL[42]. These studies, together with our findings, demonstrate that RPA-CRISPR/Cas12a assays are versatile and effective for detecting both viral genomes and more complex bacterial genomes such as *T. pallidum*.

To facilitate syphilis diagnosis in resource-limited regions and low-income countries, we integrated the RPA-CRISPR/Cas12a detection system with LFA technology, enabling point-of-care testing without reliance on large laboratory instruments. This approach enhances accessibility and scalability for field diagnostics.

Despite these advantages, several limitations of the current study should be acknowledged. First, standardized laboratory guidelines for syphilis nucleic acid testing have not yet been established, and TP-DNA detection rates vary markedly depending on sample type and timing of collection. Previous studies indicate that skin and mucosal lesion samples collected during early infection or prior to treatment such as primary chancres or secondary syphilis exudates, yield the highest detection sensitivity (approximately 50–90%) when targeting the tp47 gene[1]. In contrast, detection rates are substantially lower in whole blood or plasma (28%), urine (24%), and rectal swab samples (20%)[27]. However, lesion sampling is invasive, technically challenging, and often poorly accepted by patients. Wu et al. reported that oral rinse swab samples achieved a TP-DNA detection rate of 37.8%, with significantly higher positivity in patients exhibiting rapid plasma reagin (RPR) titers ≥ 1:32[43]. In the present study, peripheral venous blood was selected as the sample type to balance feasibility, patient compliance, and diagnostic specificity; nevertheless, optimization of sample collection timing was not systematically addressed and may have affected the overall detection rate. Second, the analytical sensitivity of LFA-based readouts is inherently lower than that of fluorescence-based detection, as the latter benefits from highly sensitive signal quantification using specialized instrumentation. Third, although RPA can be performed at room temperature, the requirement to open reaction tubes for downstream CRISPR/Cas12a processing increases the risk of aerosol contamination and false positive results. To mitigate this risk, all experiments were conducted in a standard PCR laboratory environment, and nucleic acid scavenging reagents were routinely employed for contamination control. Future development of closed-tube or one-pot reaction formats may further enhance assay robustness. Finally, the design and optimization of RPA-CRISPR/Cas12a assays remain technically demanding, requiring careful selection of primers, crRNAs, and reaction parameters. Although costs may be reduced through large-scale implementation, these considerations remain critical during assay development and clinical translation.

Conclusions

We have successfully established and validated a rapid, sensitive, and specific nucleic acid visual detection platform for *T. pallidum* based on the integration of RPA and CRISPR/Cas12a. The system supports dual readout modalities: a fluorescence-based assay and an LFA-based assay, both demonstrating high specificity and sensitivity with no observed false-positive results. The fluorescence-based method achieved a limit of detection (LOD) of 11.34 copies/μL, while the LFA-based approach exhibited an LOD of 5.56×10^2 copies/μL. Collectively, the RPA-CRISPR/Cas12a platform offers a robust, efficient, and user-friendly tool for syphilis diagnosis. Its minimal equipment requirements, rapid turnaround, and adaptability to point-of-care formats highlight its strong potential for deployment in resource-limited settings and for large-scale screening applications.

Data availability

The dataset generated and/or analyzed during the study are available from the corresponding author on reasonable request.

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Author contributions

W. L. and S. O. were responsible for conceptualizing the research. W. L., Y.S., and S. O devised the methodology. Y. S., M.Y., J.O., W.X., Y.S and X.H. carried out the formal analysis and investigation. The original draft of the manuscript was prepared by W. L., Y. S., and M.Y. Subsequently, the writing process underwent review and editing by S. O. W.L. D.N and S. O. secured the funding for this project. S. O. supervised the overall study. Each author participated in revising the manuscript and lent their approval to the final version.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The study protocol complied with the ethical guidelines of the *Declaration of Helsinki* and was approved by the Ethics Review Committee of the Ninth People's Hospital of Dongguan (Ethics Review No. 3, 2022).

Additional information

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